STUDIES ON THE ISOLATED ISLET TISSUE OF FISH. I. THE CYTOCHROME OXIDASE AND SUCCINIC DEHYDROGENASE CONTENTS OF NORMAL TOADFISH (OPSANUS TAU)

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A determination of the metabolic characteristics of the insulin-producing beta cells of the pancreas has been undertaken in the hope that these studies may offer an explanation as to why the beta cells are selectively killed by toxic agents such as alloxan (Dunn, Sheehan and McLetchie, 1943). Several hypotheses have been advanced to explain this selectivity (Lazarow, 1949; Lazarow, Liambeis and Jan Tausch, 1950) but these must be verified by direct chemical and enzymatic analyses of the insulin-producing cells. Unfortunately, it is difficult to study the metabolism of the beta cells in mammalian species because the islet tissue is inextricably intermingled with the pancreatic acinar tissue. In teleost fish, however, the insulin-secreting cells are segregated into one or more discrete bodies called the principal islet (located in the mesentery), whereas the pancreatic acinar tissue is diffusely dispersed throughout the mesentery, along the bile ducts, and within the liver (Diamare, 1899; Rennie, 1905). The isolation of insulin from the principal islet tissue of teleost fish and its absence from the diffuse acinar tissue suggested that islet tissue was the source of insulin in mammals (McLeod, 1922). Although the fish islet tissue contains several cell types (Jackson, 1922), it may be presumed by analogy to mammalian studies that it is the beta cells which produce insulin. Since alloxan diabetes and beta cell destruction has been produced in fish (Lazarow and Berman, 1947, and also unpublished observations) it may be possible to deduce information about the metabolic specialization of the beta cells themselves by comparing normal islet tissue with islets obtained from alloxan diabetic fish (*i.e.,* islets with decreased numbers of insulin producing cells).

Unfortunately the islet tissue of a 200 gm. toadfish weighs between 1 and 2 mg. and therefore it has been necessary to undertake this study by ultramicro analytical techniques. We have begun a systematic investigation of the enzymes and metabolic activities of normal fish islet tissue; the cytochrome oxidase and succinic dehydrogenase contents are herein reported. It is hoped that these studies may help to explain the mechanism by which alloxan produces selective necrosis of the beta cells, and that perhaps they may contribute to our understanding of the etiology of human diabetes. They may also furnish information useful for the cultivation of islet tissue *in vitro,* a problem which is becoming increasingly important in view of the approaching shortage of insulin.

Methods and Materials

Mature toadfish weighing between 200 and 600 gm. were kept in a running sea water tank for one to seven days prior to use. The animals were decapitated and

samples of the various organs were removed and placed in a beaker at 0° C. The principal islet was usually found near the spleen between the layers of the mesentery. The capsule of the islet was dissected and removed. The entire islet and pieces of the other organs weighing between two and 20 mg. were placed between two layers of stretched Parafilm and weighed on a Roller-Smith Micro Torsion Balance to the nearest 1/100th of a milligram.[1]

The weighed tissue was transferred to a conical-tipped micro homogenizer (Lazarow and Portis, 1951) previously cooled to 0° C. The Parafilm was reweighed, and five volumes of fluid were added to the homogenizer with a syringe buret (Lazarow, 1950). The tissues were homogenized by hand, and after appropriate dilution the enzyme contents were determined. The order in which the different tissues were assayed was varied systematically from day to day.

Succinic dehydrogenase was determined by measuring the rate of reduction of cytochrome *c* under standardized conditions in the presence of sufficient cyanide to inhibit any cytochrome oxidase present (Cooperstein, Lazarow and Kurfess, 1950). The tissue was homogenized in 5 volumes of sodium succinate (0.5 *M* in 0.2 *M* PO_4 pH 7.4) and the homogenate was diluted with the same solution to give a final tissue dilution which ranged from 1 : 20 for islet to 1 : 300 for heart. Two determinations were carried out on each sample by mixing 10 or 20 μl. of the tissue homogenate, 10 μl. of a cyanide solution and 280 μl. of a cytochrome *c*-salt solution. The extinction was followed for three minutes at 550 mμ. The enzyme activity of a standard tissue dilution was calculated as previously described (Cooperstein, Lazarow and Kurfess, 1950) and is proportional to the Δlog [ferri-cytochrome *c*]/minute. It represents the change observed in the logarithm of the concentration of oxidized cytochrome *c* per minute if one mg. of tissue is added to 100 μl. of the cytochrome *c*-cyanide solution.[2]

Cytochrome oxidase was measured by determining the rate of oxidation of reduced cytochrome *c* (Cooperstein and Lazarow, 1951). The tissues were homogenized in 5 volumes of *M*/30 phosphate buffer, pH 7.4, and diluted with the same buffer to give a final tissue dilution ranging between 1 : 20 and 1 : 150. Two or 4 μl. of the diluted tissue preparation were added to 300 μl. of the reduced cytochrome *c* solution. The enzyme activity for a standard tissue dilution was calculated and it is proportional to the Δlog [ferro-cytochrome *c*]/minute. It represents the change in the logarithm of the concentration of reduced cytochrome *c*/minute when one mg. of tissue is suspended in 100 μl. of cytochrome *c* solution.[3]

[1] Tissues included between two layers of Parafilm do not dry out (weights remain unchanged $\pm$ .02 mg. over a two-hour period).

[2] Example: When 10 μl. of a 1 : 20 islet tissue were added to 290 μl. of a cytochrome *c*-cyanide solution and the logarithms of the extinctions (corrected for the blank) at 550 mμ were plotted against time, the Δ log [cytochrome c^{+++}]/minute was equal to .088.

$$\text{The standard enzyme activity} = \frac{\Delta\log \text{ cyt. } c \times \text{dilution} \times 300}{\text{volume tissue suspension used} \times 100}$$

$$= \frac{.088 \times 20 \times 300}{10 \qquad 100} = .528.$$

[3] Calculated as in footnote 2.

Results

The average succinic dehydrogenase activity of toadfish islet and of other selected fish tissues are arranged in the order of their activity and are shown in Table I. The final tissue dilutions under the conditions of the assay are recorded, as well as the average value of 10 determinations carried out on 10 separate fish. The standard deviations [4] and the p values are also recorded. The p value recorded represents the statistical significance of each average value as compared to the tissue of next lower activity.

It was found that islet tissue is included in the group of five tissues (testis, ovary, gill and muscle) that have the lowest succinic dehydrogenase activity. The

Table I

Succinic dehydrogenase content of toadfish tissues

Tissue	No. of fish studied	Actual tissue dilution used during assay	Average standard activity $\Delta\log$ [cyto. c]/min. for a 1:100 tissue dilution	Standard deviation σ	P value compared to next lower value
Heart	10	1:9000	4.12	±0.78	<.0001
Liver	10	1:3000	1.24	±0.22	<.0001
Kidney	10	1:1500	.72	±0.17	.102
Brain	10	1:1200	.62	±0.11	<.0001
Islet	10	1:600	.26	±0.05	.88
Testis	10	1:600	.25	±0.03	.44
Gill	10	1:600	.24	±0.06	.75
Muscle	10	1:600	.23	±0.05	.60
Ovary	10	1:600	.22	±0.04	

individual values in this group are not significantly different from one another. Kidney and brain are each about 2½ times as active as islet. Liver and heart are, respectively, about 5 and 20 times as active as is islet tissue.[5]

The average cytochrome oxidase activities of tissues obtained from 10 different fish are recorded in Table II. Gill and muscle have a somewhat lower cytochrome oxidase content than does islet tissue, whereas brain, ovary and testis have a somewhat higher activity than islet (26 per cent, 23 per cent, and 44 per cent, respectively). Kidney and liver have approximately three times and heart approximately six times as much cytochrome oxidase as does islet.[5]

Cytochrome oxidase is the only known enzyme (or enzyme complex) in animal tissues involved in the biological oxidation of cytochrome *c*. By contrast, a number of dehydrogenases, including succinic dehydrogenase, are involved in the biological reduction of cytochrome *c*. It was of interest, therefore, to calculate the ratio of the enzymes which both oxidize and reduce cytochrome *c*. The succinic dehydrogenase: cytochrome oxidase ratios are plotted in Figure 1. Although the value of this ratio is very slightly greater for islet tissue than for ovary or testis,

[4] $\sqrt{\frac{\Sigma(\text{difference})^2}{N}}$.

[5] The relative enzyme activities for different tissues are unchanged when the results are converted to a dry weight basis.

TABLE II

Cytochrome oxidase content of toadfish tissues

Tissue	No. of fish	Final tissue dilution used in assay	Average standard activity Δlog [cyto. *c*]/min. for 1:100 tissue dilution	Standard error σ	P value compared to next lower tissue
Heart	10	1:22,500	10.5	1.97	<.0001
Kidney	10	1:15,000	6.06	0.84	.76
Liver	10	1:15,000	5.96	0.72	<.0001
Testis	10	1:6,000	2.64	0.25	.028
Brain	10	1:6,000	2.31	0.39	.76
Ovary	10	1:6,000	2.27	0.25	<.0001
Islet	10	1:4,500	1.83	0.21	<.0001
Gill	10	1:3,000	1.40	0.21	.0010
Muscle	10	1:3,000	1.12	0.15	

the ratio of succinic dehydrogenase (which reduces cytochrome *c*) to cytochrome oxidase (which oxidizes cytochrome *c*) is less in islet tissue than in any of the other tissues studied with the exception of kidney. Even though the succinic dehydrogenase: cytochrome oxidase ratio in toadfish kidney is somewhat lower than in islet tissue, the absolute amount of succinic dehydrogenase present was almost three times that of the islet tissue.

In order to determine whether the enzyme studies carried out on the islet tissue of fish may be extrapolated to mammalian islet tissues where direct enzyme study is difficult, we have compared the enzyme contents of corresponding tissues of the

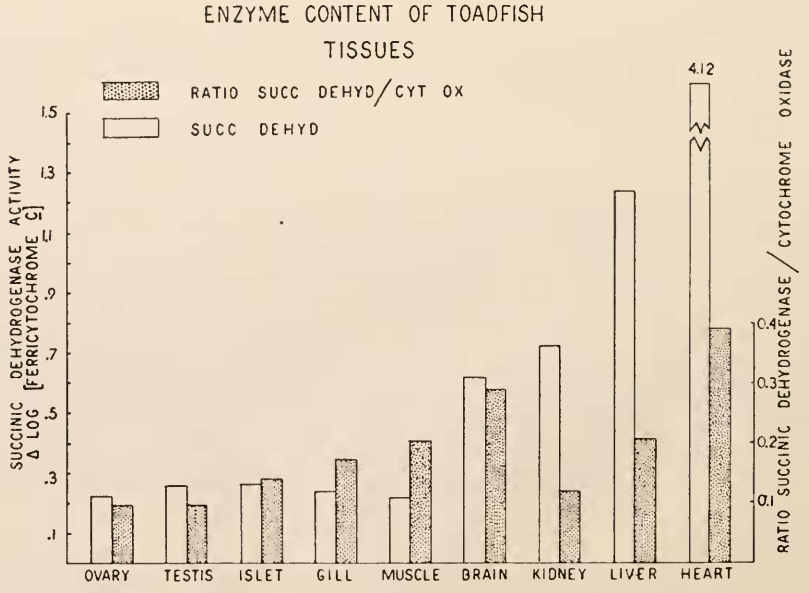

FIGURE 1.

rat and the toadfish. The enzyme activity of the most active tissue in each species (heart) is arbitrarily set at 100 per cent and the activity of the other tissues is expressed in terms of their relative activity (Table III).

Although the absolute enzyme activity of rat heart is about three times as great as toadfish heart, the relative enzyme concentrations in the corresponding tissues show good agreement in most cases. The toadfish kidney is an exception in that it contains only 18 per cent as much succinic dehydrogenase as toadfish heart, whereas rat kidney is 58 per cent as active as rat heart.

Discussion

Dunn, Sheehan and McLetchie showed in 1943 that alloxan selectively killed the beta cells in the islets of Langerhans of the pancreas. Although the exact mechanism by which alloxan destroys the pancreatic beta cells has remained obscure, it is apparent that alloxan is a selective rather than a specific poison, since larger doses

Table III

Relative enzyme content of tissues of toadfish and rat

	Cytochrome oxidase		Succinic dehydrogenase	
	Rat	Toadfish	Rat	Toadfish
Heart	100%	100%	100%	100%
Kidney	71	58	58	18
Liver	36	57	42	30
Brain	19	22	15	15
Muscle	12	11	6	6

of this compound will also produce necrosis of the liver and kidney cells (Dunn and McLetchie, 1943; Goldner and Gomori, 1943; Palay and Lazarow, 1946).

Several mechanisms have been suggested to explain the selectivity of alloxan for the beta cells (Lazarow, 1949). The metabolism of the beta cell differs from other cells in that it is continually synthesizing insulin. Insulin is a protein which has an unusually high content of sulfur amino acids (12 per cent cystine) (Du Vigneaud, 1927). Since all the sulfur of insulin is in the disulfide form, it might be expected that the metabolism of a cell synthesizing insulin would be geared to the oxidation of sulfhydryl groups to their disulfide form. Since sulfhydryl compounds such as glutathione and cysteine have been shown to protect rats against alloxan diabetes (Lazarow, 1946), and since cysteine is also used for the synthesis of glutathione, it has been postulated that the specialization of the beta cell for insulin synthesis may deplete the beta cell gluthathione and thereby increase the susceptibility of these cells to alloxan diabetes (Lazarow, 1949).

Alloxan is destroyed in the body by several reactions, one of which is its reduction to a non-diabetogenic derivative (Lazarow, Patterson and Levey, 1948), dialuric acid. This reduction, which can be accomplished by glutathione and by cysteine, can also be carried out by the reduced coenzyme, diphosphopyridine nucleotide (Cooperstein, Lazarow and Patterson, 1951). By contrast, dialuric acid can be re-oxidized to alloxan by cytochrome *c* (Cooperstein, Lazarow and Patterson,

1951) and by methylene blue (Lazarow and Liambeis, 1950). The potentiation of alloxan diabetes by methylene blue has been attributed to the reoxidation of any dialuric acid formed *in vivo* back to its diabetogenic derivative, *i.e.*, alloxan (Lazarow and Liambeis, 1950).

A further consequence of the specialization of the beta cells for insulin synthesis may be a decreased ability to destroy alloxan. If the metabolism of a cell is geared to the oxidization of sulfhydryl to disulfide, this cell may also be less able to reduce alloxan to dialuric acid (or it may rapidly reoxidize any dialuric acid formed back to alloxan). The finding by Bensley (1911) that islet tissue reduces Janus Green more slowly than does acinar tissue is in keeping with this hypothesis.

The enzyme studies reported here may likewise lend support to this hypothesis. Since dialuric acid is oxidized to an active diabetogenic compound, *i.e.*, alloxan, by the oxidized form of cytochrome *c*, it is apparent that the concentration of the oxidized form of this enzyme would play a role in determining the effective concentration of alloxan in the beta cell. The concentration of oxidized cytochrome *c* would in turn be determined by (a) the total concentration of cytochrome *c* present, by (b) the relative concentration of the enzymes which both oxidize and reduce cytochrome *c* and by (c) the spatial relations of these enzymes. Whereas the oxidation of cytochrome *c* is accomplished only by the cytochrome oxidase complex, the reduction of cytochrome *c* can be accomplished by a number of dehydrogenases. The succinic dehydrogenase complex is only one of the enzymes capable of reducing cytochrome *c*. It is apparent that both the absolute concentration of succinic dehydrogenase as well as the relative concentrations of succinic dehydrogenase to cytochrome oxidase in the beta cell would undoubtedly influence the fraction of cytochrome *c* that is in the oxidized state.

However, since other cytochrome-reducing systems would also play a role, it will be necessary to have a more complete picture of other enzymes in the islet tissue.

Since islet tissue was found to be low in succinic dehydrogenase and to have a low succinic dehydrogenase to cytochrome oxidase ratio (Fig. 1) (*i.e.*, it contains a relative abundance of cytochrome oxidase), it would appear that if the distribution of other cytochrome reducing enzymes parallels that of succinic dehydrogenase, islet tissue may well have a relatively high concentration of oxidized cytochrome *c* within the cell. Therefore, following the injection of a given dose of alloxan, any dialuric acid formed by the *in vivo* reduction of alloxan would be re-oxidized more readily and the effective concentration of alloxan within the beta cells would be higher. These cells might therefore be more susceptible to destruction by alloxan than would be the case with other tissues such as heart, liver, kidney, brain, and possibly muscle. On the basis of these enzyme studies, however, the gonads should be equally susceptible to alloxan. It must be remembered that on the other hand, the blood supply of an organ also determines the concentration of alloxan reaching that organ. The extremely rapid rate at which alloxan is destroyed does not permit equilibration to take place. On the basis of their blood supply, very vascular organs such as the islet tissue, liver, and kidney, might be expected to receive a higher total dose of alloxan than would be the case with the less vascular organs such as gonad or muscle.

It is of considerable interest that the relative enzyme activity of most of the tissues examined, using the enzyme content of the heart as 100 per cent, is the same

for rat and toadfish organs. This would suggest that the comparisons between the islet and other tissues of the toadfish may also reflect the enzyme status in mammalian tissue where direct analysis of islet tissue is more difficult. Toadfish kidney, however, differs from the other tissues studied in that it contains comparatively less succinic dehydrogenase than does rat kidney. The toadfish kidney also differs morphologically from rat kidney in that the former is aglomerular and it contains a large amount of lymphoid tissue. Whether this striking difference in enzyme content is a reflection of the differences in secretory mechanisms of glomerular and aglomerular kidneys, the presence of lymphoid tissues, the marine habitat, or the presence of gills which may take over a part of the renal excretory function, awaits further investigation.

Determinations of these enzymes in islet tissue with diminished numbers of beta cells (alloxan diabetic) will be carried out at a later date.

Summary

1. The succinic dehydrogenase and cytochrome oxidase contents of toadfish islet tissues have been determined by measuring spectrophotometrically the rate of reduction and oxidation of cytochrome *c* under standardized conditions.

2. The succinic dehydrogenase content of islet tissue was much lower than heart, liver, kidney or brain, and of the same order of activity as testis, ovary, muscle and gill.

3. The ratio of succinic dehydrogenase (a cytochrome *c*-reducing enzyme) to cytochrome oxidase (the cytochrome *c*-oxidizing enzyme) in islet tissues was low when compared to muscle, brain, liver and heart.

4. The relation of these findings to the selective destruction of the pancreatic beta cells by alloxan has been discussed.

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